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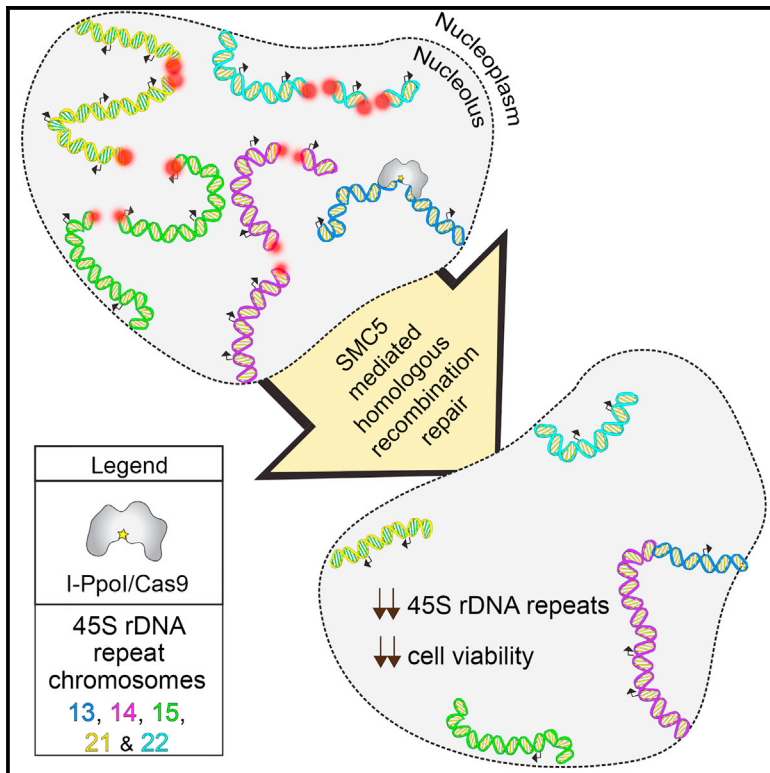
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# Cell Reports

## Breaks in the 45S rDNA Lead to Recombination-Mediated Loss of Repeats

### Graphical Abstract



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### In Brief

rDNA constitutes the largest repetitive sequence in the human genome. Warmerdam et al. show that cells are highly sensitive to breaks in 45S, but not 5S, rDNA repeats. Since 45S repeats are distributed over multiple chromosomes, this complicates recombination-mediated repair, leading to a loss of repeats and cellular viability.

### Highlights

- Human cells are highly sensitive to breaks in 45S, but not 5S, rDNA repeats
- Homologous recombination inhibits break repair in 45S rDNA
- SMC5 contributes to recombination-mediated repair of rDNA breaks
- Recombination-driven repair of 45S rDNA results in loss of repeats and viability



# Breaks in the 45S rDNA Lead to Recombination-Mediated Loss of Repeats

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## SUMMARY

rDNA repeats constitute the most heavily transcribed region in the human genome. Tumors frequently display elevated levels of recombination in rDNA, indicating that the repeats are a liability to the genomic integrity of a cell. However, little is known about how cells deal with DNA double-stranded breaks in rDNA. Using selective endonucleases, we show that human cells are highly sensitive to breaks in 45S but not the 5S rDNA repeats. We find that homologous recombination inhibits repair of breaks in 45S rDNA, and this results in repeat loss. We identify the structural maintenance of chromosomes protein 5 (SMC5) as contributing to recombination-mediated repair of rDNA breaks. Together, our data demonstrate that SMC5-mediated recombination can lead to error-prone repair of 45S rDNA repeats, resulting in their loss and thereby reducing cellular viability.

## INTRODUCTION

In order to maintain genomic stability, cells contain a variety of quality control systems that ensure accurate transmission of their genome during cell division. In addition, cells can detect damage to the genome and promote repair or cell death (Hoeijmakers, 2009; Jackson and Bartek, 2009). DNA double-stranded breaks (DSBs) severely threaten genomic stability. Cells are protected from breaks by the DNA damage response, a signaling cascade activating the DNA damage checkpoints and responsible for the initiation of DNA repair. DSBs can be repaired through direct ligation of broken DNA ends in a process called non-homologous end joining (NHEJ) and through homologous recombination (HR). The latter process requires an undamaged homologous sequence present on another undamaged homologous sequence—usually, the sister chromatid (Warmerdam and Kanaar, 2010). Unlike NHEJ, HR-mediated repair is thought to be an error-free process.

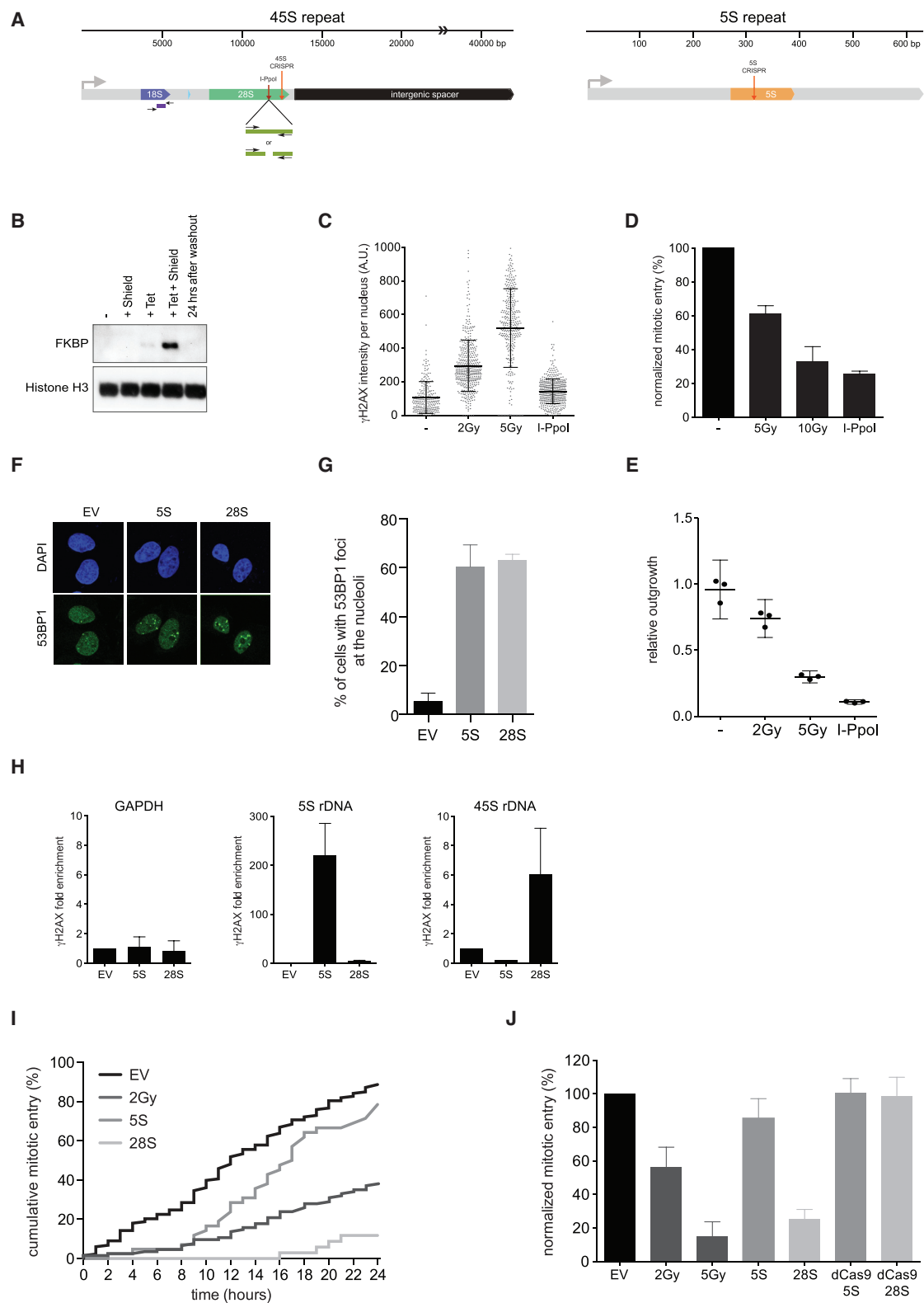
rDNA is the largest protein-coding repeat in the genome. In *S. cerevisiae*, loss of active repeats enhances genome instability, resulting in reduced proliferation and accelerated aging (Ide

et al., 2010; Kobayashi, 2006, 2014), underlining the importance of maintaining rDNA stability for genomic integrity and cellular viability (Kobayashi et al., 2004). A recent report indicated that breaks in the 45S repeat are predominantly repaired through NHEJ (Harding et al., 2015). However, HR-mediated repair is also involved in the restoration of breaks in the rDNA (Torres-Rosell et al., 2005b, 2007; van Sluis and McStay, 2015). Moreover, the rDNA is a recombinational hotspot in cancer, and translocations involving the rDNA are one of the most commonly observed chromosomal alterations in solid tumors (Stults et al., 2009). In yeast, the inhibition of DNA resection inhibits rDNA recombination (Hoang et al., 2010; Tan et al., 2012), and, in humans, DNA breaks in highly transcribed regions are more likely to become repaired through HR (Aymard et al., 2014). This would suggest that recombination-mediated repair could facilitate repair in the human rDNA locus. However, the repetitive nature of the rDNA and its distribution over multiple chromosomes make recombination-mediated repair of broken rDNA potentially error prone. Here, we investigate how human cells cope with breaks within the rDNA repeats. Interestingly, cells are highly sensitive to breaks in 45S repeats as compared to breaks in the 5S rDNA repeats. We found that HR perturbs the repair of breaks in the 45S rDNA and produces a reduction in the number of repeats, combined with reduced cellular viability.

## RESULTS AND DISCUSSION

### Targeting the 45S rDNA Repeats

To investigate how human cells deal with breaks in rDNA repeats, we made use of the I-Ppol nuclease that cuts a rare sequence present in the 45S rDNA locus of human cells (Figure 1A), next to approximately 30 sites present elsewhere in the human genome (Berkovich et al., 2007; Monnat et al., 1999). Using a switchable system in which I-Ppol was fused to the FKBP destabilization domain and placed under the response of a tetracyclin-inducible promoter, we can turn I-Ppol activity on and off through the addition or removal of tetracyclin and shield-1 (Figure 1B), allowing us to generate a limited amount of breaks and follow their repair. We optimized I-Ppol induction in U2OS cells to produce levels of H2AX phosphorylation ( $\gamma$ H2AX), a well-characterized marker for DSBs, comparable to 2 Gy of ionizing radiation (IR) (Figures 1C, S1A, and S1B).



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Next, we examined checkpoint activation upon induction of breaks in the rDNA. Activation of I-Ppol-induced breaks in G2 cells resulted in an 80% reduction in mitotic entry, compared to undamaged cells (Figures 1D and S1C). In contrast, breaks generated through 5 and 10 Gy IR resulted in a 40% and 75% reduction in mitotic entry, indicating that breaks in the rDNA lead to a more prominent G2 arrest. We found similar results in retinal pigment epithelium (RPE) cells (Figure S1D). As expected, I-Ppol-induced G2 checkpoint activation was largely dependent on the DNA damage checkpoint kinase ATM (ataxia telangiectasia mutated) (Figures S1E–S1G). In addition, colony outgrowth in U2OS and RPE cells was severely impaired by I-Ppol-induced breaks, and also when compared to breaks generated through IR, indicating that cells do not easily cope with damage in the rDNA repeats (Figures 1E and S1H).

### Breaks in the 45S rDNA Transiently Inhibit rDNA Transcription

rDNA repeats are clustered in nucleolar organizer regions (NORs) (Richard et al., 2008; Sakai et al., 1995) in the nucleolus, a specialized compartment within the nucleus that enables the transcription and subsequent production of the ribosome. Polymerase-I-dependent transcription of the 45S repeats in active NORs produces a 45S pre-rRNA that is processed to 28S, 5.8S, and 18S transcripts. The 5S repeat is transcribed by polymerase III, producing the 5S transcript. Combined, these four transcripts are part of the 60S (28S, 5.8S, and 5S) and 40S (18S) ribosomal subunits (Tschochner and Hurt, 2003). Besides harboring the rDNA, the nucleolus also acts as a central hub for signaling pathways that are important for cell proliferation and genomic integrity (Boisvert et al., 2007; Hein et al., 2013). When analyzing 45S pre-rRNA and 18S transcript levels following rDNA breaks, we did not find a reduction in the abundance of these transcripts 2 and 24 hr after I-Ppol induction (Figure S2A). Treatment of cells with 5 Gy IR also did not reduce rRNA levels, whereas inhibition of polymerase I using actinomycin D did result in a transient reduction in 45S pre-rRNA transcript levels. I-Ppol induction did produce a transient inhibition in rDNA transcription, as determined by incorporation of 5-ethynyl uridine in

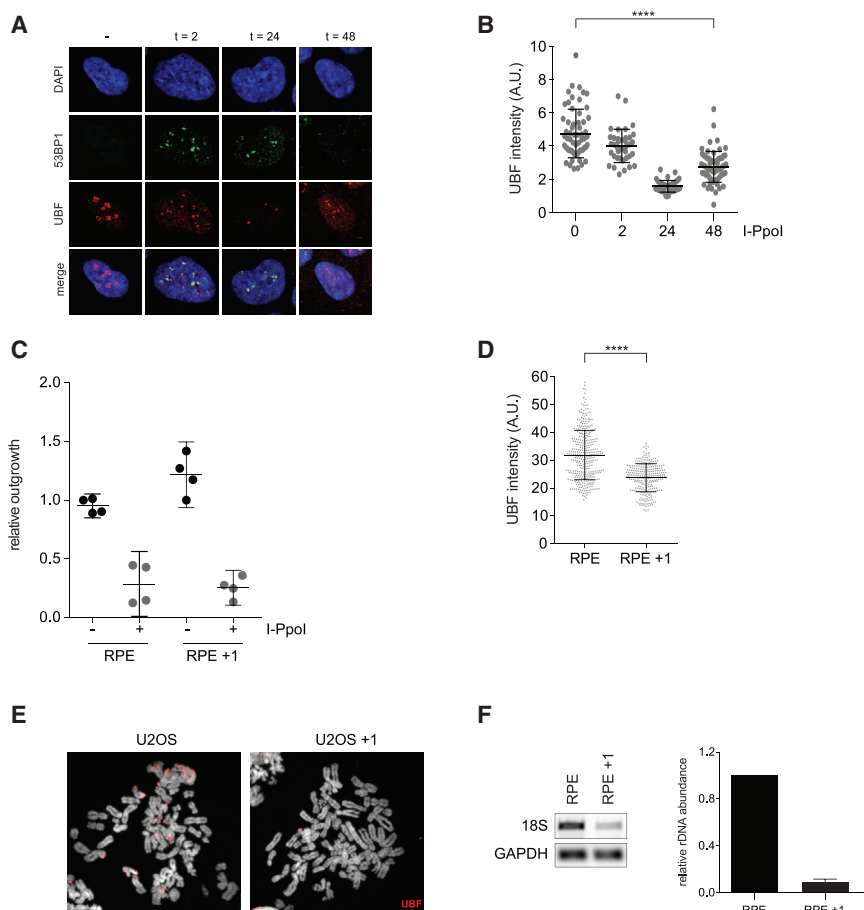
the nucleoli (Figures S2B and S2C). Inhibition of rDNA transcription was dependent on ATM (Harding et al., 2015; Kruhlak et al., 2007; van Sluis and McStay, 2015) (Figures S2B and S2C), and inhibition of rDNA transcription is known lead to a re-localization of nucleolar components to the nucleoplasm (Burger et al., 2010). Indeed, we found that nucleolin and nucleophosmin transiently translocate out of the nucleoli after I-Ppol induction (Figures S2D–S2F) in an ATM-dependent manner (Figure S2B), consistent with previous findings (Harding et al., 2015). Thus, we established a tunable rDNA break system that transiently inhibits rDNA transcription but does not seem to impair 45S transcript levels.

### Cells Are Less Sensitive to Breaks in the 5S Than in the 45S rDNA Repeat

The rDNA is located on the 45S and 5S loci. The 45S locus consists of approximately 300 repeats distributed over the five acrocentric chromosomes. The 5S locus contains a similar amount of repeats uniquely located on chromosome 1 (Gibbons et al., 2015). To investigate whether cells respond similarly to breaks in the 45S and 5S rDNA repeat, we used CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (Cong et al., 2013; Mali et al., 2013), in combination with guides targeting a sequence in either the 45S or 5S rDNA (Figures 1A and S1J). Breaks introduced in the rDNA of RPE cells result in recruitment of 53BP1, a known DNA damage response protein, into large foci that localize close to the nucleolar periphery (Figure 1F), consistent with recent evidence that damaged rDNA relocates to the nucleolar periphery (Harding et al., 2015; van Sluis and McStay, 2015). We observed 53BP1 foci at the nucleolar periphery after the induction of Cas9 with either of the two rDNA targeting guides in 60% of the cells, whereas only 5% of these cells were observed in the control (Figure 1G), indicating that the 5S and 45S guides specifically generate breaks in the rDNA. Indeed, using chromatin immunoprecipitation (ChIP) with an antibody directed against  $\gamma$ H2AX, we could show that activation of Cas9 produces a clear enrichment of  $\gamma$ H2AX at the designated target location only (Figure 1H). Interestingly, the increase in H2AX phosphorylation appears to be greater at the 5S repeats, compared to the 45S repeats.

### Figure 1. Cells Are More Sensitive toward Breaks in the 45S than in the 5S rDNA Repeat

- (A) Graphical illustration of 45S and 5S rDNA repeats, with the location of the I-Ppol and CRISPR target sites and primers used.
- (B) U2OS cells stably expressing FKBP-HA-I-Ppol were synchronized by thymidine and released in normal medium for 8 hr in the presence of tetracyclin (Tet) and shield-1 (Shield) for the last 1 hr. Cells were washed and lysed after 1 or 24 hr and analyzed by western blot with the indicated antibodies.
- (C) U2OS I-Ppol cells were synchronized in G2 and damaged using I-Ppol, 2 Gy IR, or 5 Gy IR. After 1 hr, cells were fixed for immunofluorescence. The average  $\gamma$ H2AX intensity per nucleus was determined in at least 250 cells. Error bars represent SD.
- (D) Same as in (C), but cells were damaged using I-Ppol, 5 Gy IR, or 10 Gy IR. After 1 hr, cells were washed, and medium-containing nocodazole was added. Cells were collected after 24 hr, fixed and stained for MPM2 and propidium iodide (PI), and analyzed by flow cytometry. The undamaged control was normalized to 100%. Error bars represent the SEM of three independent experiments.
- (E) Colony survival of U2OS I-Ppol cells damaged with I-Ppol, 2 Gy IR, or 5 Gy IR. Error bars represent 95% confidence interval (CI).
- (F) RPE cells expressing Cas9 were infected with CRISPR guides targeting the 5S or 45S rDNA. After 24 hr, cells were fixed for immunofluorescence and stained against 53BP1.
- (G) Quantification of (F). Error bars represent the SEM of three independent experiments. EV, empty vector.
- (H) Same as in (F), but after 24 hr, cells were lysed. ChIP for  $\gamma$ H2AX was performed, followed by qPCR with the indicated primers. Error bars represent SD of three technical replicates.
- (I) RPE cells expressing Cas9 and mCherry-53BP1 were followed directly after infection with the guide sequences using live-cell imaging. For 5S and 28S, cells containing 53BP1 foci were followed, and entry into mitosis was determined over time. At least 100 cells were counted for each condition.
- (J) Same as in (I), but 24 hr after transfection, nocodazole was added for an additional 24 hr. Cells were fixed and stained for MPM2 and PI. Error bars represent the SEM of three independent experiments.



**Figure 2. Breaks in the 45S rDNA Result into a Loss of Repeats**

(A) RPE I-Ppol cells were synchronized in G2, damaged using I-Ppol, pre-extracted, and fixed for immunofluorescence at the indicated time points. Cells were stained with the indicated antibodies.

(B) Quantification of the UBF in (A). Each dot represents a cell. Error bars represent SD. \*\*\*\*p < 0.0001.

(C) Colony survival of RPE and RPE+1 cells after I-Ppol activation. Error bars represent 95% CI.

(D) RPE and RPE+1 cells were pre-extracted and fixed for immunofluorescence. Each dot represents a cell. Error bars represent SD. \*\*\*\*p < 0.0001.

(E) Chromosome spreads of U2OS and U2OS+1 cells. After fixation, cells were stained against UBF. Representative images are shown.

(F) Genomic DNA was extracted from RPE and RPE+1 cells. GAPDH and 18S were amplified using PCR. Error bars represent SEM of three independent experiments.

Using time-lapse microscopy, we observed that breaks in 5S resulted in an 8-hr delay in mitotic entry (Figure 1I), whereas only a very small percentage of cells recovered after targeting of the 45S rDNA repeats. Trapping cells in mitosis by the addition of nocodazole confirmed that breaks in the 45S rDNA induce a much stronger G2 arrest than those in the 5S rDNA (Figure 1J). Cas9 activity was required to induce breaks, since expression of the guides in combination with a nuclease-deficient Cas9 did not arrest the cells (Figures 1J and S1J). Together, these results indicate that human cells are more sensitive to DSBs in the 45S than in the 5S rDNA repeat.

### Loss of 45S rDNA Repeats after Damage

The distribution of the 45S rDNA repeats over multiple chromosomes could, of course, complicate recombination-mediated repair, resulting in an increase or decrease of repeats. Therefore, we investigated the abundance of repeats after the introduction of rDNA breaks. Upstream binding factor (UBF) binds the rDNA promoter region and localizes inside the nucleoli (Wright et al., 2006). After I-Ppol activation, UBF relocates toward the outside of the nucleoli co-localizing with the damage marker 53BP1 (van Sluis and McStay, 2015) (Figure 2A). UBF levels were clearly decreased at 24 hr after I-Ppol activation (Figure 2B), at a time when the majority of cells still contained breaks, as observed by the presence of 53BP1 foci. UBF levels

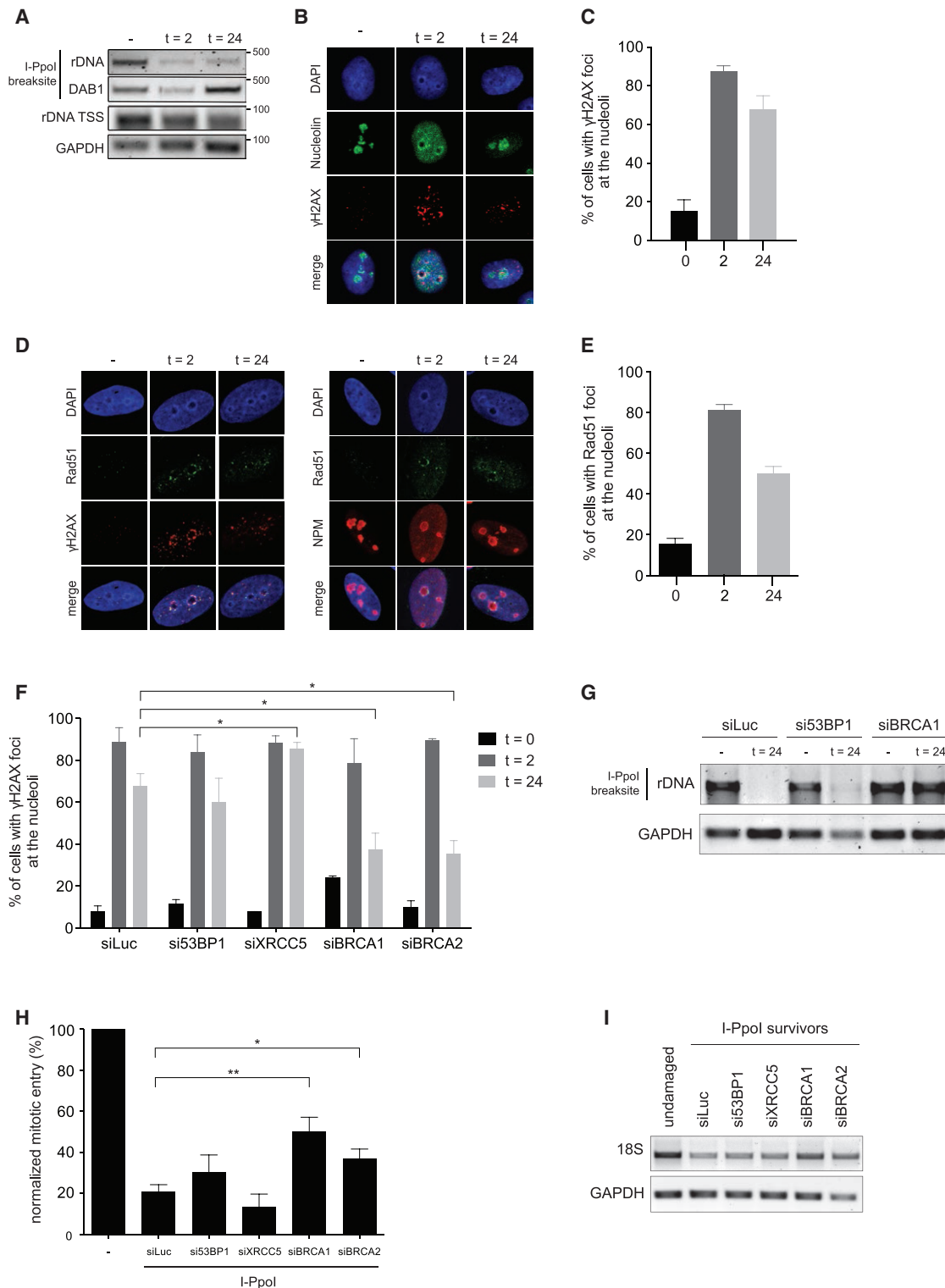
were partly restored after 48 hr but remained lower than before the damage, also in cells without 53BP1 foci, indicating that the amount of rDNA was decreased after the completion of repair. Interestingly, cells that survived I-Ppol activation, indicated as RPE+1 (or U2OS+1), remained sensitive to I-Ppol-induced damage, as determined by colony outgrowth (Figure 2C), indicating

that at least a subset of the I-Ppol target sites, and thus part of the rDNA repeats, were maintained. Also, UBF protein levels in the nucleus remained similar after I-Ppol activation (Figure S2G), whereas UBF levels at the nucleoli decreased in RPE+1 cells (Figure 2D). A reduction in UBF was also observed on the chromosomes of U2OS+1 cells when compared to the naive cell lines, suggesting that rDNA break survivors have a lower amount of rDNA repeats (Figure 2E). Indeed, compared to the undamaged control cells, RPE+1 cells display a large reduction in the abundance of 18S rDNA (Figure 2F). Combined, these results clearly indicate that rDNA repeats can be lost after damage, likely because of incorrect alignments between the rDNA repeats on sister chromatids or between the repeats on separate chromosomes.

### HR-Mediated Repair of 45S rDNA Breaks

Besides the 45S rDNA, I-Ppol also cuts at a location in the DAB1 gene on chromosome 1 (Goldstein et al., 2013). Using PCR and primer pairs designed at opposite sides of the break (Figure 1A), we observed that, 2 hr after I-Ppol activation, both the 45S and DAB1 sites were broken (Figure 3A). However, within 24 hr, the DAB1 break site was repaired, whereas the 45S rDNA sites were still largely unrepaired. Similar results were obtained in both U2OS and RPE cells (Figure S3A), indicating that breaks in the 45S rDNA are not repaired efficiently. To further investigate





**Figure 3. Recombination Hampers Repair of 45S rDNA Breaks Causing Loss of Repeats**

(A) Genomic DNA was extracted from G2-synchronized U2OS I-Ppol cells 2 and 24 hr after I-Ppol activation. PCR was conducted using primers located at the I-Ppol break site in the 45S and DAB1 loci, the rDNA transcriptional start site (TSS), and GAPDH.  
(B) U2OS I-Ppol cells were synchronized in G2 and fixed for immunofluorescence after I-Ppol activation. Samples were stained with the indicated antibodies.  
(C) Quantification of (B) showing the percentage of cells containing nucleolar γH2AX foci. Error bars represent SEM of three independent experiments.

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rDNA break formation and repair, we determined the amount of cells with  $\gamma$ H2AX foci close to the nucleoli at different time points after I-Ppol induction (Figures 3B, 3C, and S3B). The presence of  $\gamma$ H2AX at the rDNA was confirmed by ChIP (Figure S1I). We observed that the intensity of  $\gamma$ H2AX foci decreased over time (Figure S3C), but we found that a large fraction of the introduced rDNA breaks were not efficiently repaired, as determined by the persistence of  $\gamma$ H2AX foci close to the nucleoli 24 hr after I-Ppol activation (Figures 3C and S3B). We confirmed that breaks in 45S are not repaired efficiently, by determining the residence time of 53BP1-mCherry foci at 5S, 45S, and IR-induced break sites (Figure S1L).

To investigate whether breaks in the 45S repeat can also be subject to recombination-mediated repair, we determined the recruitment of HR-related repair proteins, such as Rad51, RPA, BRCA1, and BRCA2 into I-Ppol-induced foci. We found that all of these are recruited to foci that overlap with  $\gamma$ H2AX and localize at the nucleolar periphery after the induction of breaks in the rDNA (Figures 3D, 3E, and S3G), indicating that breaks in the rDNA can also form a substrate for homology-directed repair. We confirmed these results after generating breaks using the 5S and 28S CRISPR guides (Figure S1K). We found that Rad51, RPA, and BRCA1 all localize at the nucleolar periphery, together with  $\gamma$ H2AX. Combined, these results suggest that HR is active at rDNA breaks.

### Resolution of 45S rDNA Breaks Is Hampered by HR

HR-mediated repair of the rDNA repeats after incorrect alignment could result in gain or loss of repeats. Moreover, inter-chromosomal repair through HR could result in chromosomal translocations. To determine how classical end joining and recombination-mediated repair influenced the resolution of rDNA breaks, we inhibited either pathway through siRNA (small interfering RNA)-mediated depletion of 53BP1, XRCC5, BRCA1, or BRCA2 (Figure S3D). We determined  $\gamma$ H2AX foci formation and resolution at 2 and 24 hr after I-Ppol induction (Figure 3F). Phosphorylation of H2AX was dependent on ATM, as expected (Figure S3E). Knockdown of any of the genes did not particularly alter normal cell-cycle distribution (Figure S3F). However, depletion of BRCA1 and BRCA2 significantly enhanced the resolution of I-Ppol-induced  $\gamma$ H2AX foci, whereas the amount of breaks that were introduced was similar to those of the control (Figure 3F). In contrast, the depletion of XRCC5 decreased the resolution rDNA breaks, indicating that inhibition of NHEJ can result in the processing of rDNA breaks through HR. Loss of 53BP1 did not produce a similar shift, but since 53BP1 has been shown to play a role in recombination-driven repair of breaks during G2 (Kakarougkas et al., 2013), it may affect

both pathways of repair in G2 cells. Similar results were observed in RPE cells after the loss of 53BP1 and BRCA1 (Figure S3H). To determine repair of the rDNA after I-Ppol more directly, we monitored the break site by PCR and could show that loss of BRCA1 increases the repair of rDNA breaks (Figure 3G). Combined, these results indicate that the resolution of rDNA breaks is troubled by HR-directed repair.

To confirm this, we inhibited HR-mediated repair through the addition of a specific Rad51 inhibitor. This efficiently repressed Rad51 filament formation, a requirement for successful strand invasion (Figure S4A) (Kanaar et al., 2008) and increased the resolution of  $\gamma$ H2AX foci (Figure S4B), again indicating that inhibition of HR results in more efficient repair of rDNA breaks. Consistent with this notion, loss of HR increased mitotic entry of G2 cells by 20%–40%, whereas the loss of XRCC5 resulted in a 10% decrease, compared to the control (Figure 3H). Similar results were found in RPE cells after the loss of BRCA1 (Figure S3I). Thus, loss of HR can partially rescue the rDNA-break-induced G2 arrest, strengthening the conclusion that HR-mediated repair hinders the repair of broken rDNA repeats.

Loss of HR through the depletion of BRCA2 or the inhibition of Rad51 leaves resected breaks. However, our results indicate that these ends are efficiently processed and repaired. Possible explanations for this observation are that the produced overhangs form a substrate for classical NHEJ, because the nuclease activity of Artemis can trim them down to directly fusible ends (Ma et al., 2002; Riballo et al., 2004), or that resected ends form a substrate for repair through alternative NHEJ, which utilizes micro-homology (Sfeir and Symington, 2015). Indeed, proteins involved in micro-homology-mediated end joining localize to the nucleoli (Medler et al., 2005). However, it should also be considered that the DNA damage response in the nucleolus might be different from what we know happens elsewhere in the nucleus (Boulon et al., 2010).

To investigate whether recombination is responsible for the loss of repeats after damage in the rDNA, we determined the abundance of rDNA in cells that survived I-Ppol induction and were inhibited for either NHEJ or HR (Figure 3I). Depletion of BRCA1 and BRCA2 rescues the rDNA-break-induced reduction in rDNA, indicating that HR is, indeed, responsible for the loss of rDNA repeats during repair.

### Loss of SMC5 Enhances Cell Viability after Breaks in the 45S rDNA

In yeast cells, DSBs in the rDNA can be repaired through HR, and *smc5* facilitates this process (Torres-Rosell et al., 2005b, 2007). Because SMC5 is conserved throughout evolution and has been implicated in DNA repair in higher eukaryotes (Jeppsson et al.,

(D) Same as in (B). Samples were stained with the indicated antibodies.

(E) Quantification of (D) showing the percentage of cells containing nucleolar Rad51 foci. Error bars represent SEM of three independent experiments.

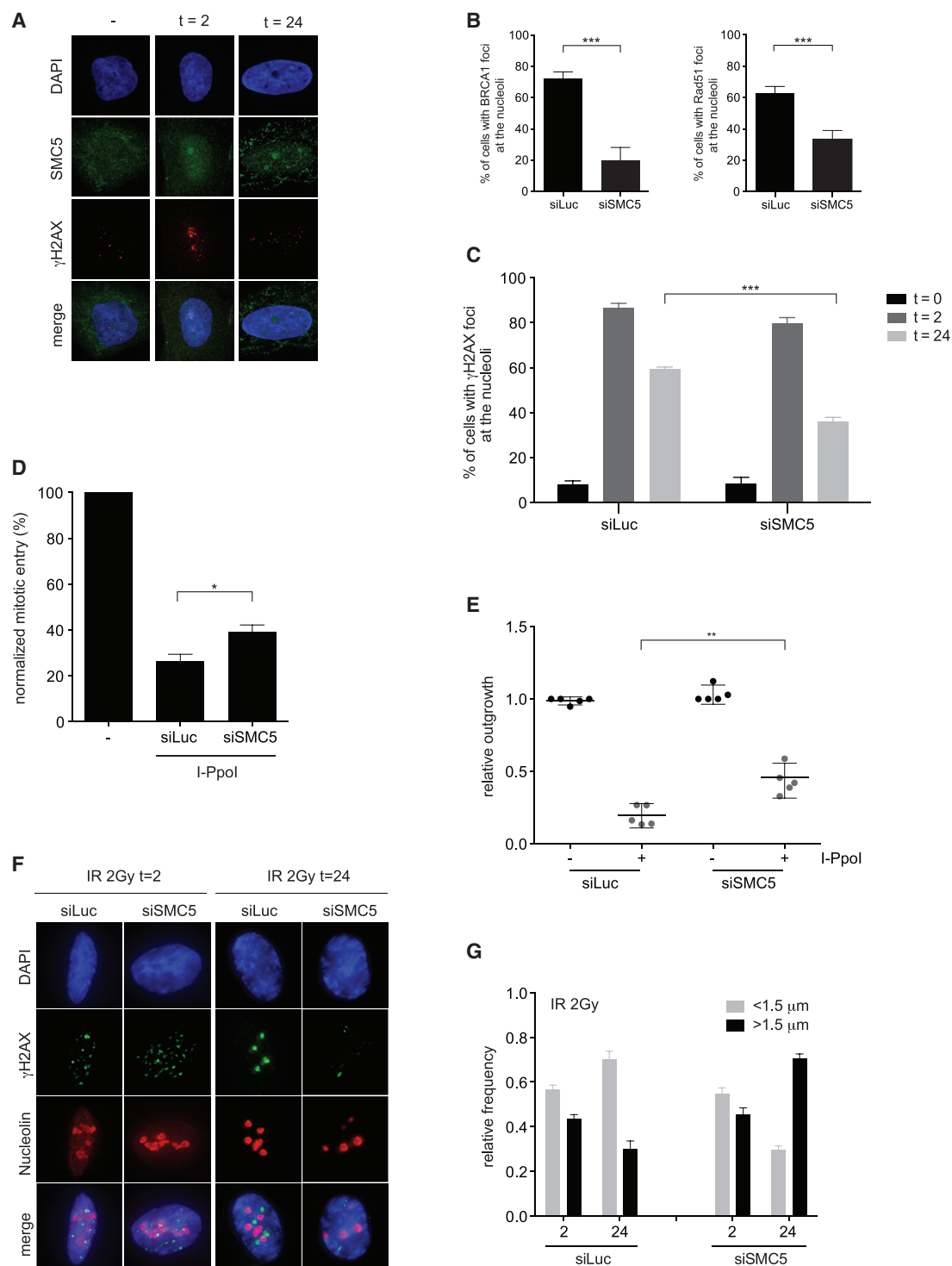
(F) U2OS I-Ppol cells were transfected with siRNAs against Luciferase (siLuc), 53BP1 (si53BP1), XRCC5 (siXRCC5), BRCA1 (siBRCA1), and BRCA2 (siBRCA2) and synchronized in G2. 36 hr later, I-Ppol was activated, and cells were fixed for immunofluorescence at the indicated time points. The percentage of cells containing nucleolar  $\gamma$ H2AX foci was determined. Error bars represent the SEM of three independent experiments. \* $p < 0.05$ .

(G) Same as in (A), but here, RPE I-Ppol cells were transfected with the indicated siRNAs. Genomic DNA was PCR amplified using 45S and GAPDH primers.

(H) Same as in (F), but here, cells were incubated with nocodazole for 24 hr. Cells were fixed and stained for MPM2 and PI. Error bars represent the SEM of three independent experiments. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

(I) Genomic DNA was extracted from RPE+1 cells that were depleted for the indicated proteins. Genomic DNA was PCR amplified using 18S and GAPDH primers.





**Figure 4. SMC5 Mediates Recombination-Mediated Repair of Breaks in the 45S rDNA**

(A) RPE I-Ppol cells were synchronized in G2, damaged using I-Ppol, fixed for immunofluorescence, and stained with the indicated antibodies. (B) U2OS I-Ppol cells were transfected with siRNAs against Luciferase (siLuc) or SMC5 (siSMC5). Cells were fixed for immunofluorescence and stained. The percentage of cells containing nucleolar BRCA1 and Rad51 foci was determined. Error bars represent the SEM of three independent experiments. \*\*\* $p < 0.001$ . (C) RPE I-Ppol cells were transfected with siRNAs against Luciferase or SMC5 and synchronized in G2. 36 hr after transfection I-Ppol was activated and cells were fixed for immunofluorescence at the indicated time points. Error bars represent the SEM of three independent experiments. \*\*\* $p < 0.001$ .

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2014), we investigated the function of this protein in the repair of rDNA breaks in human cells (Potts et al., 2006). Interestingly, SMC5 relocates to the nucleoli after I-Ppol-induced breaks, suggesting that SMC5 is involved in the repair of rDNA breaks (Figure 4A). Consistent with a role in HR, we found a significant reduction in BRCA1 and Rad51 recruitment to foci after loss of SMC5 (Figures 4B, S4C, and S4D). Also, loss of SMC5 resulted in enhanced resolution of  $\gamma$ H2AX foci after I-Ppol activation, suggesting that SMC5 facilitates the repair of rDNA breaks through HR (Figure 4C; Figure S4E). The absence of SMC5 also resulted in more recovery (Figure 4D), indicating that SMC5-mediated repair blocks cell-cycle re-entry after rDNA breaks. Importantly, loss of SMC5 partially rescued the reduction in survival after I-Ppol activation (Figure 4E), in line with our observation that recombination-mediated loss of repeats reduces cell viability.

One shortcoming of the systems we used thus far is that they all result in the induction of many breaks in the rDNA repeats. To investigate whether rDNA breaks induced by IR are similarly difficult to resolve, we irradiated cells with a low dose of IR. Interestingly, we found that  $\gamma$ H2AX foci that are in close proximity to the nucleoli appear to be more difficult to resolve, as evidenced by their persistence at later time points (Figure S4F). Loss of SMC5 reduced the persistence of the IR-induced nucleolar  $\gamma$ H2AX foci, as compared to the control (Figures 4F, 4G, and S4F), indicating that SMC5 also plays a role in the repair of IR-induced rDNA breaks. Combined, these results identify SMC5 as an important modulator involved in the repair of broken rDNA. Interestingly, yeast without *smc5* is genomic unstable, and mutations in SMC5 are found in human cancers (Torres-Rosell et al., 2005a), suggesting that deregulation of SMC5 could be a cause of genomic instability in humans as well.

Taken together, we present an inducible rDNA break system that allows us to study how human cells respond to breaks in the rDNA. We show that, unlike breaks in the 5S rDNA, breaks in the 45S rDNA are not repaired efficiently. Unresolved 45S rDNA breaks result in prolonged checkpoint activation and reduced cell viability. Breaks in the 45S rDNA are a substrate for recombination-mediated repair, and, as a result, cells lose repeats. We identify SMC5 as a specific regulator of HR-mediated rDNA repair, and we show that loss of recombination promotes more efficient repair of rDNA breaks, promotes maintenance of rDNA integrity, and enhances cell viability. Although HR is commonly thought to represent an error-free process, our results indicate that it can also be error prone. It will be interesting to investigate whether other repeats in our genome are also subject to this type of error-prone recombination-regulated repair.

## EXPERIMENTAL PROCEDURES

Immunofluorescence, flow cytometry, western blotting, and ChIP were performed as described previously (Medhurst et al., 2008; Schmidt et al., 2009;

van Vugt et al., 2004; Warmerdam et al., 2009). Cell culture and PCR were performed using standard methods. Detailed protocols can be found in the [Supplemental Experimental Procedures](#). Plasmids, siRNAs, inhibitors, antibodies, and primer sequences used in this study are described in the [Supplemental Experimental Procedures](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.02.048>.

## AUTHOR CONTRIBUTIONS

D.O.W. and R.H.M. designed the study. D.O.W. and J.v.d.B. carried out and analyzed experiments. D.O.W. and R.H.M. wrote the manuscript.

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(D) Same as in (C), but here, nocodazole was added for 24 hr after I-Ppol activation. Cells were fixed and stained for MPM2 and PI. Error bars represent the SEM of three independent experiments. \* $p < 0.05$ .

(E) Colony survival of RPE I-Ppol cells depleted for Luciferase or SMC5 before and after I-Ppol activation. Error bars represent 95% CI. \*\* $p < 0.01$ .

(F) RPE I-Ppol cells were depleted for Luciferase or SMC5, synchronized in G2, and fixed for immunofluorescence 2 and 24 hr after 2 Gy IR. Samples were stained with the indicated antibodies.

(G) Quantification of (F). The distance of a focus to the closest nucleolus was measured. The frequency of  $\gamma$ H2AX foci closer or further away than 1.5  $\mu$ m from the nucleoli was determined. Error bars represent the SEM of three independent experiments.

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